Hypertriglyceridemia but not diabetes status is associated with VLDL containing apolipoprotein CIII in patients with coronary heart disease

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Abstract

High apoCIII concentration in apoB lipoproteins is a prominent component of atherogenic dyslipidemia, and explains the risk of coronary heart disease (CHD) associated with high triglyceride (TG). We hypothesized that diabetic people have atherogenic dyslipidemia with apoCIII in excess of that accounted for by their high TG levels. We selected 30 diabetic and 30 nondiabetic persons, 15 of each with fasting TG < 160 mg/dl and 15 with TG ≥ 200 mg/dl. Using immunoaffinity chromatography and ultracentrifugation, we prepared large and small VLDL, IDL and LDL with or without apoCIII or apoE. The groups with TG ≥ 200 mg/dl, regardless of diabetes status, had higher concentrations of large and small VLDL particles with apoCIII and higher apoCIII concentrations than the groups with fasting TG < 160 mg/dl. The diabetes groups did not have higher concentrations of these lipoproteins than the nondiabetes groups within the same fasting TG criteria. In conclusion, high concentrations of apoCIII-containing VLDL are associated with hypertriglyceridemia, which may play a critical role in identifying the high risk of CHD in hypertriglyceridemic patients whether diabetic or nondiabetic. Diabetes status per se does not appear to be associated with high concentrations of apoCIII-containing TG-rich lipoprotein particles, if the plasma TG levels are similar.

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1. Introduction

Diabetic patients have higher plasma triglyceride (TG) concentrations than nondiabetics, and considerably higher rates of coronary heart disease (CHD). It is not entirely clear whether the high TG concentration contributes independently to CHD in diabetes. In the Paris Prospective Study, TG concentration was an independent predictor in people with impaired glucose tolerance or diabetes even after adjustment for HDL-C and other risk factors [1]. However, in the much larger UKPDS, high TG was significant only in univariate but not in multivariate analysis that included HDL-C [2]. VLDL and IDL concentrations are high in diabetic patients [3] due to a high VLDL production rate [4–6] and a slow clearance of VLDL [7,8]. The plasma concentration of apolipoprotein CIII (apoCIII) is elevated in diabetic patients [9,10], and is positively correlated with TG levels [11]. ApoCIII delays metabolism of TG-rich particles by inhibiting lipoprotein lipase activity [12–14] and apoE-dependent receptor binding [15–18], which increases the probability of deposition of cholesterol of lipoprotein particles in the vessel wall. It is possible that the plasma TG concentration does not capture the full adverse effect on CHD of abnormal TG and VLDL metabolism in diabetic patients.

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The plasma level of apoCIII in apoB lipoproteins is an independent risk factor for CHD [19,20], and for worsening of coronary atherosclerosis [21], and in one cross-sectional study, it was associated with atherosclerotic vascular disease in NIDDM [22]. In multivariate analysis, apoCIII in VLDL + LDL was a much stronger risk factor than TGs for coronary events, and accounted for the univariate association between TGs and coronary events [19]. It is possible that apoCIII may be especially important in diabetes, since the apoCIII gene has an insulin response element [23,24] which decreases apoCIII production. Since the plasma levels of apoCIII in apoB lipoproteins are strongly correlated with fasting TG levels [11,19,25], it has not been clearly shown whether patients with diabetes have higher plasma levels of apoCIII than patients without diabetes, if their TG levels are similar. A higher apoCIII concentration could help explain the high incidence of CHD in diabetic people. Therefore, we hypothesized that plasma concentrations of apoB lipoproteins with apoCIII, (apoC-III+) would be higher in diabetic than non-diabetic patients with CHD, and help explain the higher rate of CHD in diabetics. To test this hypothesis, we divided both diabetic and non-diabetic patients into two groups based on their fasting TG levels and compared the particle concentrations of apoCIII-containing apoB lipoproteins between diabetic and nondiabetic groups with similar TG levels.

In addition to apoCIII, we considered the role of apoE in diabetic dyslipidemia. Metabolically, apoE contributes to clear VLDL and LDL particle from plasma, functioning as a primarily ligand for both LDL receptor [26] and LDL receptor-related protein [27]. Thus, we would expect that the apoE in VLDL would be a protective influence against CHD. Surprisingly, apoE level in apoB lipoproteins is high in CHD [19,20,28–30]. Since most of apoE coexists with apoCIII in humans [31,32], apoE on apoB particles may simply be a marker for the presence of apoCIII. Therefore, we isolated apoB containing lipoproteins based on the presence of both apoE and CIII to define more precisely the role of each apolipoprotein in diabetic dyslipidemia.

2. Materials and methods

2.1. Subjects and study design

We selected 60 patients from the population in the Cholesterol and Recurrent Events (CARE) trial. The CARE trial was a randomized, double-blinded, placebo-controlled trial of pravastatin in 4159 patients who had myocardial infarction (MI) 3–20 months prior to enrollment in which 14% (N = 586) of patients had diabetes at baseline [33]. The study design and the characteristics of diabetic patients were described previously [34,35]. In brief, inclusion criteria were cholesterol < 240 mg/dl, LDL cholesterol 115–174 mg/dl, fasting TG levels < 350 mg/dl, and fasting glucose levels < 220 mg/dl. Institutional Review Boards of the centers approved the study, all patients gave informed consent, and the procedures were in accordance with institutional guidelines. For the present study, the diabetic patients were identified by a self-reported history of diabetes and a fasting blood glucose ≥ 126 mg/dl. Nondiabetic patients were identified by an absence of history of diabetes and a fasting glucose < 110 mg/dl. The diabetic and nondiabetic groups were divided into two groups on the basis of their fasting TG either ≥ 200 mg/dl (HTG) or < 160 mg/dl (NTG). Thus, four groups were established; diabetes with hypertriglyceridemia (DM-HTG), diabetes with normal triglycerides (DM-NTG), nondiabetic with hypertriglyceridemia (nonDM-HTG), and nondiabetic with normal triglycerides (nonDM-NTG). We placed a gap between the criteria for normal and high TG levels to amplify the distinction between the groups. Age (5-year interval) and sex were matching factors among the four groups. Patients who were taking sulfonylurea or acarbose were eligible for inclusion, whereas those taking metformin, insulin or medications affecting lipid metabolism including estrogen, progesterone, beta-blockers and diuretics were excluded. Since plasma from the baseline period of the trial was used in this study, no patients were taking lipid-lowering medications at the time of blood sampling.

2.2. Preparation of blood samples

Fasting blood was sent by overnight delivery in cold containers to the core laboratory in St. Louis, MO. Plasma was prepared by centrifugation at 4 °C; 1-ml aliquots were stored in polypropylene tubes at -80 °C. Vials containing frozen plasma were shipped on dry ice by overnight delivery from the core laboratory to the Harvard School of Public Health in Boston. Analyses were conducted with four samples at a time, including one subject from each of the four groups. None of the personnel at the laboratories were aware of the group identification. The code was maintained at the Data Coordinating Center, University of Texas School of Public Health, Houston. Each vial was thawed immediately at 37 °C for 5 min and the plasma samples were separated by immunoaffinity chromatography followed by ultracentrifugation.

2.3. Immunoaffinity chromatography

We separated plasma into four immunofractions with two-step incubation first with anti-apoE (Perimmune, Inc., Rockville, MD) and second with anti-apoCIII (DMA, Arlington, TX) coupled to Affigel resin. The
Fasting Plasma

Anti-apoE Immunoaffinity Chromatography

E+ (The bound)  E- (The unbound)

Anti-apoCIII Immunoaffinity Chromatography

E+CIII+  E+CIII-  E-CIII+  E-CIII-

Ultrancentrifugation
VLDL-large (60<Sf<200), VLDL-small (20<Sf<60), IDL (1.006<d<1.025), and LDL (1.025<d<1.050)

Lipids and Apolipoprotein Measurements:
TG, Cholesterol, ApoB, ApoCIII, and ApoE

Fig. 1. Summary of laboratory procedures. 1 ml of fasting plasma was incubated with anti-apoE and anti-apoCIII immunoaffinity chromatography in sequence to isolate four types of lipoproteins: E+CIII+, E−CIII+, E+CIII−, and E−CIII−. Each immunofraction was ultracentrifuged to separate large VLDL, small VLDL, IDL, and LDL, then lipids and apolipoproteins were measured.

preparation of resins and validation of the immunoaffinity chromatography procedures were described previously [31]. Fig. 1 shows the flowchart of the immunoaffinity separation. In brief, plasma was incubated at 4 °C overnight with anti-apoE resin, the unbound lipoproteins that were poor in apoE (E−) were collected by gravity flow, and the resin was washed with 5 ml of phosphate buffered-saline (PBS). The bound fractions, lipoproteins that were rich in apoE (E+), were eluted with two separate incubations with 1 ml of 3 M sodium thiocyanate (NaSCN), the first incubation for 3 min and the second incubation for 1.5 min, respectively. NaSCN contained in the E+ fractions was removed immediately by gel filtration (PD-10, Amersham-Pharmsacia Biotech, Sweden). The E+ and E− fractions were subsequently incubated with anti-apoCIII immunoaffinity chromatography using the same incubation, elution, and desalting conditions described for anti-apoE immunoaffinity chromatography. As a result, four immunofractions were isolated from the plasma of each patient (E+CIII+, E+CIII−, E−CIII+, and E−CIII−). These plasma fractions were concentrated to 0.6 ml with Ultrafree concentrators (molecular size cutoff, 50 kDa, Millipore, Bedford, MA). For simplicity, we did not report the results for the E+CIII− fraction, which were very low in all subjects as we reported previously for nondiabetic people [31,32]. The recoveries after immunoaffinity chromatography were 87% for cholesterol and 83% for TG.

2.4. Very fast ultracentrifugation

For ultracentrifugation, the following equipment was used: Beckman Optima TLX ultracentrifuge, Beckman TLA 120.2 fixed-angle rotor (r_max = 38.9 mm, r_min = 24.5 mm), thick-wall polycarbonate tubes (11 x 35 mm, No. 343778, without cap, Beckman, Palo Alto, CA). After each centrifugation, the supernatants were collected by aspiration with a glass Pasteur pipette (Fischer, Pittsburgh, PA). Large VLDL (60<Sf<200), small VLDL (20<Sf<60), IDL (1.006<d<1.025 kg/l), and LDL (1.025<d<1.050) were isolated by stepwise increase of the solute density through the addition of potassium bromide solutions (KBr, SIGMA, St. Louis, MO) with the method by Pietzsch et al. [36] with the following modifications. Densities were adjusted and verified on an optical densitometer (Bausch & Lomb, Rochester, NY). To prepare large VLDL, we transferred 600 μl of the concentrated immunofraction to an ultracentrifugation tube. The sample was overlaid with 400 μl of d = 1.006 kg/l and submitted to ultracentrifugation (23 min, 15 °C, 157 000 x g). The large VLDL in the top 400 μl of each tube was carefully harvested. To prepare small VLDL, the remaining solution was carefully mixed by pipetting, overlaid with 400 μl of d = 1.006 kg/l, and submitted to ultracentrifugation (1 h 17 min, 15 °C, 537 000 x g). The small VLDL was drawn with the same manner described for the large VLDL. To prepare IDL, we mixed 35.9 μl of 40% saturated KBr solution with 64.1 μl of double distilled water and the 100 μl of mixture was added to the remaining fraction to adjust the density to 1.025 kg/l. The final volume was made up to 1 ml with d = 1.025 kg/l. Centrifugation was performed (1 h 51 min, 15 °C, 537 000 x g) and 400 μl of the supernatant, containing IDL, was collected. To prepare LDL, we mixed 52.1 μl of 40% saturated KBr solution with 47.9 μl of double distilled water and added the 100 μl of mixture to the remaining fraction to adjust the density to 1.050 kg/l. After making the final volume up to 1 ml with d = 1.050 kg/l, we submitted the sample to ultracentrifugation (2.5 h, 15 °C, 537 000 x g). 400 μl of supernatant containing LDL, was collected. We used d < 1.025 for IDL to collect all possible IDL particles and used d < 1.050 for LDL to isolate relatively pure LDL, minimizing contamination with lipoprotein (a) and large-size HDL particles. The run time and speeds were standardized by comparison with the classic Lindgren method using a SW41 swinging bucket rotor (Beckman, Palo Alto, CA) so that the lipid measurements of the two ultracentrifuge methods were similar as previously shown [37]. It was shown that this ultracentrifuge method only minimally
affects apolipoproteins of these lipoprotein particles [36].

2.5. Lipid and apolipoprotein measurements

ApoB was measured by sandwich ELISA. The following materials were used: goat polyclonal anti-human apoB (Biogenesis Co., Rockville, MD, coating antibody) and mouse monoclonal anti-human apoB (Chemicon International Inc., Temecula, CA, capture antibody), alkaline phosphatase-linked conjugate (Jackson Immunoresearch Lab Inc., Westgrove, PA) and p-nitrophenyl phosphate (PNPP) substrate (Sigma Chemicals), and 3 M NaOH solution for stopping color development. The plate was read at 405 nm. Standards were calibrated to reference plasma provided by the Centers for Disease Control (Atlanta, GA). Cholesterol and TG were measured by enzymatic methods (Cobas MIRA plus, Roche Diagnostic System Inc., Nutley, NJ). ApoCIII and apoE were measured by immunoturbidimetry (WACO chemicals USA, Richmond, VA) on the Cobas MIRA plus system. Intra-assay coefficient of variance of cholesterol, TG, apoB, apoCIII, and apoE were 2, 3, 6, 5 and 5%, respectively. Inter-assay coefficient of variance for measurements were 4, 6, 8, 10 and 7%, respectively. Plasma insulin was measured using microparticle enzyme immunoassay (Abbott Laboratories, Tokyo, Japan) at the core laboratory for the CARE trial, Washington University in St. Louis. This method was highly selective for insulin with no cross-reactivity with proinsulin (<0.005%).

2.6. Statistical analysis

Statistical analyses were performed with SAS software (Version 8.0, SAS Institute, GA). Outlier analysis was performed and no data were excluded. Analysis of variance (ANOVA) and paired group comparisons were performed with the PROC GLM CONTRAST procedure. Since one sample from each of the four groups was included in each run and since the study was completed in 15 runs, the variable 'run' was included to adjust for run-to-run variation. Multiple linear regression analysis was performed to investigate the association of apoCIII containing lipoproteins with plasma TG, HOMA index (homeostasis model assessment = glucose (mmol/l) × insulin (µU/ml)/22.4), and diabetes status. Plasma TG was dichotomized (NTG, if TG < 160 mg/dl, HTG, if TG > 200) for regression analysis. HOMA index was also added as a dichotomized variable based on the cut point for normal and insulin resistant status (0, if HOMA ≤ 2, normal; 1, if HOMA > 2, insulin resistant). BMI, history of hypertension, total cholesterol, exercise, current smoking, and use of oral hypoglycemic medications were covariates. In certain analyses, plasma glucose or insulin concentrations were added together with the other covariates instead of the HOMA index.

3. Results

3.1. Baseline characteristics

Clinical and biochemical characteristics of the patients are shown in Table 1. The TG concentrations in the HTG groups were approximately two times higher than those in the NTG groups but were similar between the two HTG groups or the two NTG groups. The concentrations of LDL cholesterol were similar in the four groups. The concentrations of HDL cholesterol were lower in the HTG groups than in the NTG groups. Groups with diabetes had higher insulin levels than the nondiabetic groups (14.6 vs. 9.1 µU/ml for DM and nonDM group, respectively, P = 0.03). The DM-HTG group had the highest insulin level and HOMA index, followed by the DM-NTG group and the nonDM-HTG group. The nonDM-NTG group had the lowest insulin levels and the lowest HOMA index. Age and sex were the matching factors and were similar among the four groups. Characteristics affecting plasma lipid levels such as alcohol consumption, smoking, and use of antihypertensive medications were similar among the four groups. BMI was higher in the DM groups than in the nonDM-NTG groups. The trend for waist circumference was in the same direction as BMI. The NTG groups in both DM and nonDM group exercised more frequently than the HTG groups.

3.2. Lipids and apolipoproteins of TG-rich lipoproteins containing apoCIII

3.2.1. Effects of hypertriglyceridemia

The effects of hypertriglyceridemia were tested by two comparisons: within diabetic groups (DM-NTG vs. DM-HTG) and non-diabetic groups (nonDM-NTG vs. nonDM-HTG). HTG status was associated with higher TG, cholesterol, apoB (Table 2), and apoCIII concentrations (Fig. 2) in large and small VLDL particles that contained apoCIII, in both diabetic and nondiabetic groups. HTG status was also associated with higher apoE concentrations in large VLDL E+CIII+ in patients with and without DM (e.g. 4.91 and 3.94 mg/dl for DM-HTG and DM-NTG, respectively, P < 0.05). The HTG groups tended to have higher concentrations of cholesterol and TG in the IDL CIII+ particles (Table 2). There was no difference in LDL concentrations between HTG and NTG groups (data not shown). ApoCIII/B ratio tended to be higher in HTG than NTG groups, although not significantly (Table 3). ApoE/B ratio was similar among the four groups (Table 3).
3.2.2. The effects of diabetes status

The effect of diabetes status on TG-rich lipoproteins was compared in normotriglyceridemic groups (DM-NTG vs. nonDM-NTG) and in hypertriglyceridemic groups (DM-HTG vs. nonDM-HTG). Diabetes status was not uniformly associated with the plasma apoB or apoCIII concentrations of apoCIII-containing (apoC-III+) lipoproteins (Table 2, Fig. 2). In no instance was an apoB lipoprotein concentration significantly higher in a DM than a nonDM group. In several instances in the HTG groups, the DM group had even lower concentrations of apoCIII+ lipoproteins than those in the nonDM group; cholesterol of E+CIII+ type of small VLDL (1.66 vs. 1.98 mg/dl, \( P = 0.02 \)), apoE of E+CIII+ type of small VLDL (1.10 vs. 1.32 mg/dl, \( P = 0.03 \)). Both ApoCIII/B and ApoE/B ratios were similar between DM and nonDM groups (Table 3).

3.3. Linear regression analysis

We performed multivariate linear regression analysis to identify the independent variables associated with apoCIII containing particles. The data for all four groups were pooled. The results were similar to those presented for the between-group comparisons in Table 2 and Fig. 2. Plasma total TG concentration was strongly associated with cholesterol and apoB levels in large VLDL E−CIII+ and small VLDL E−CII+ (e.g. \( \beta = 3.00, \ P = 0.01 \) for cholesterol of large VLDL E−CII+). Diabetes status was not associated with higher levels of apoCIII+ particles, but was associated with low levels of cholesterol and apoB in small VLDL E−CIII+ (\( \beta = -0.6, \ P = 0.006 \)). The HOMA index was associated with low levels of cholesterol in small VLDL E−CIII+ (\( \beta = -2.5, \ P = 0.053 \)). Insulin level was not...
Table 2
Lipid and apolipoprotein B concentrations

<table>
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<tr>
<th></th>
<th>DM-NTG, TG &lt; 160</th>
<th>DM-NTG, TG ≥ 200</th>
<th>NonDM-NTG, TG &lt; 160</th>
<th>NonDM-NTG, TG ≥ 200</th>
<th>ANOVA P-value</th>
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<tr>
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<td>N = 15</td>
<td>N = 15</td>
<td>N = 15</td>
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<td>VLDL-large E + CIII+</td>
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<tr>
<td>ApoB</td>
<td>2.31 ± 1.08</td>
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<tr>
<td>Cholesterol</td>
<td>5.15 ± 2.25</td>
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<td>TG</td>
<td>952.68 ± 24.04</td>
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<td>VLDL-small E + CIII+</td>
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<tr>
<td>ApoB</td>
<td>2.87 ± 1.78</td>
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<tr>
<td>Cholesterol</td>
<td>5.26 ± 2.74</td>
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<td>TG</td>
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<tr>
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<tr>
<td>ApoB</td>
<td>0.63 ± 0.28</td>
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<tr>
<td>TG</td>
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<td>IDL E + CIII+</td>
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<tr>
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<tr>
<td>Triglyceride</td>
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<td>Triglyceride</td>
<td>3.47 ± 2.23</td>
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</table>

Values are mean ± S.D. NS denotes nonsignificance at P = 0.05. † 0.05 < P < 0.1 compared with nonDM-NTG.
** P < 0.05 compared with DM-NTG.
* 0.05 < P < 0.1 compared with DM-NTG.
†† P < 0.05 compared with nonDM-NTG
‡ 0.05 < P < 0.10 compared with DM-NTG.
* 0.05 < P < 0.10 compared with DM-HTG.
** P < 0.05 compared with DM-HTG.

significantly associated with any of the apoB lipoprotein types.

4. Discussion

We previously reported that apoCIII concentrations in apoB lipoproteins are considerably stronger risk factors than plasma TG, and, in multivariate analysis, explained the mild risk associated with TG [19]. The specific role of apoCIII in diabetic patients is complicated by a moderate correlation with plasma TG concentration, a relatively weak lipid risk factor with uncertain independence as a risk factor in diabetics [1,2]. The higher TG levels in diabetics could not, per se, account for the higher CHD incidence. For example, the difference in plasma TG between diabetic and nondiabetic people found in the UKPDS [2], corresponds to a 20% increased risk in men and 40% in women, using the univariate relationship between plasma TG and CHD in a meta-analysis [38]. These estimates are halved in multivariate analysis. We aimed to evaluate the specifi-
city of involvement of apoCIII in diabetes by matching diabetic and nondiabetic patients for plasma TG concentrations. ApoCIII was associated with plasma TG concentration, as expected. However, contrary to our hypothesis, VLDL, IDL and LDL with apoCIII were similar in diabetic and non-diabetic groups. The HTG groups, diabetic or nondiabetic, had higher concentrations of VLDL with apoCIII than NTG, as previously found in other nondiabetic persons [32].

There is much evidence that apoCIII in apoB lipoproteins is an important component of atherogenic dyslipidemia. Since apoCIII inhibits lipolysis [12–14]...
and apoE-mediated clearance [15–18] of apoB lipoproteins compared with the lipoproteins that do not contain apoCIII; vascular endothelium has a high probability to take up these particles. This mechanism may be similar in patients with and without diabetes. It has been suggested that apoCIII may have a particular role in patients with diabetes, since insulin resistance or insulin deficiency in diabetic patients blunts downregulation of apoCIII expression in the liver [23,24]. Diabetic patients usually have higher levels of apoCIII and plasma TG than controls [39–41]. Dallongeville et al. demonstrated that polymorphism in the insulin response element of the apoCIII gene promoter influences the correlation between insulin and TG or TG-rich lipoproteins in humans [42]. Both VLDL TG and apoCIII production are increased by the liver and by cultured hepatocytes of obese, hyperinsulemic rats [43,44]. ApoCIII production is increased in patients with hypertriglyceridemia [45], although it is unknown whether this is dependent or independent of glycemic or insulimic status. In the present study, we did not find a relationship between fasting insulin and VLDL apoB or apoCIII concentrations, although the sample size may have limited the ability to detect mild correlations. However, it has not been clearly shown that plasma apoCIII concentrations are higher in patients with diabetes if their TG levels are matched with patients without diabetes. The present study found that the plasma concentration of VLDL particles that contain apoCIII and the apoCIII content of these particle types are strongly associated with fasting TG levels, not with diabetes status per se. This agrees with the report by Ishibashi et al. [46] that the apoCIII content of VLDL was similar in diabetics and nondiabetics. This suggests that mechanisms that establish hypertriglyceridemia and atherogenic dyslipidemia may be similar in patients with and without diabetes.

In conclusion, our findings suggest that fasting TG level is a key determinant for apoCIII concentrations in TG-rich lipoproteins, regardless of diabetes status. Although it is likely that these TG-rich lipoproteins contribute to the high incidence of CHD in patients with diabetes, diabetes status per se does not appear to be associated with high concentrations of apoCIII-containing TG-rich lipoprotein particles, if their plasma TG levels are similar.

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